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(54) Title: **SELF-CONTAINING LACTOCOCCUS STRAIN**

(57) Abstract: The invention relates to a recombinant *Lactococcus* strain, with environmentally limited growth and viability. More particularly, it relates to a recombinant *Lactococcus* that can only survive in a medium, where well-defined medium compounds are present. A preferred embodiment is a *Lactococcus* that may only survive in a host organism, where said medium compounds are present, but cannot survive outside the host organism in absence of said medium compounds.

WO 02/090551 A2

**SELF-CONTAINING *Lactococcus* STRAIN****Field of the invention**

The invention relates to a recombinant *Lactococcus* strain, with environmentally limited growth and viability. More particularly, it relates to a recombinant *Lactococcus* that can only survive in a medium, where well-defined medium compounds are present. A preferred embodiment is a *Lactococcus* that may only survive in a host organism, where said medium compounds are present, but cannot survive outside the host organism in absence of said medium compounds. Moreover, said *Lactococcus* can be transformed with prophylactic and/or therapeutic molecules and can, as such, be used to treat diseases such as inflammatory bowel diseases.

**Background of the invention**

Lactic acid bacteria have long time been used in a wide variety of industrial fermentation processes. They have generally-regarded-as-safe status, making them potentially useful organisms for the production of commercially important proteins. Indeed, several heterologous proteins, such as Interleukin-2, have been successfully produced in *Lactococcus* spp (Steidler *et al.*, 1995). It is, however, unwanted that such genetically modified micro organisms are surviving and spreading in the environment. To avoid unintentional release of genetically modified microorganisms, special guidelines for safe handling and technical requirements for physical containment are used. Although this may be useful in industrial fermentations, the physical containment is generally not considered as sufficient, and additional biological containment measures are taken to reduce the possibility of survival of the genetically modified microorganism in the environment. Biological containment is extremely important in cases where physical containment is difficult or even not applicable. This is, amongst others, the case in applications where genetically modified microorganisms are used as live vaccines or as vehicle for delivery of therapeutic compounds. Such applications have been described e.g. in WO 97/14806, which discloses the delivery of biologically active peptides, such as cytokines, to a subject, by recombinant non-invasive or non-pathogenic bacteria. WO 96/11277 describes the delivery of therapeutic compounds to an animal – including humans – by administration of a recombinant bacterium, encoding the therapeutic protein. Steidler *et al.* (2000) describe the treatment of colitis by administration of a recombinant *Lactococcus lactis*, secreting interleukin-10. Such a

usage of a self-containing and transform d *Lactococcus* to deliver prophylactic and/or therapeutic molecules in order to prevent and/or treat diseases.

### Brief description of the figures

5 **Figure 1:** Map of the MG1363 *thyA* locus

**Figure 2:** Schematic representation of the different expression modules as present on pOThy plasmids and genomic integrants of hIL-10. Black parts represent original *L. lactis* MG1363 genetic information, white parts represent recombinant genetic information.

10 **Figure 3:** PCR identification of Thy11 (Thy11 1.1 and Thy11 7.1 represent individually obtained, identical clones). Standard PCR reactions were performed by using aliquots of saturated cultures of the indicated strains as a source of DNA template. Panel A shows an agarose gel of the products of the indicated PCR reactions. Panel B shows the positions at which primers attach in the *thyA* (1), upstream (2) or downstream (3) PCR's. Oligonucleotide primers used: (1): ATgACTTACgCAGATCAAgtTTTT and TTAATTgCTAAATCAAATTTCAATTg (2): TCTgATTgAgTACCTTgACC and gCAATCATAATTggTTTTATTg (3): CTTACATgACTATgAAAATCCg and cTTTTTTATTATTAgggAAAgCA.

20 **Figure 4:** PCR identification of Thy11, Thy12, Thy15 and Thy16. Standard PCR reactions were performed by using three days old colonies of the indicated strains as a source of DNA template.

Panel A shows the positions at which primers attach in the upstream (1), downstream (2) or *thyA* (3), PCR's. Oligonucleotide primers used: (1): ATgACTTACgCAGATCAAgtTTTT and TTAATTgCTAAATCAAATTTCAATTg (2): TCTgATTgAgTACCTTgACC and gCAATCATAATTggTTTTATTg (3): CTTACATgACTATgAAAATCCg and cTTTTTTATTATTAgggAAAgCA

Panel B shows an agarose gel of the products of the indicated PCR reactions.

30 **Figure 5:** Southern blot analysis of the indicated strains. Chromosomal DNA was extracted and digested with the indicated restriction enzymes. Following agarose gel electrophoresis the DNA was transferred to a membrane and the chromosome structure around the *thyA* locus was revealed by use of DIG labelled *thyA* or hIL-10 DNA fragments (panel A). Panel B shows a schematic overview of the predicted structure of the *thyA* locus in both MG1363 and Thy11.

diluted in TFM or TFM supplemented with 50µg/ml of thymidine (T50). CFU counts were determined at different time points: t=0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 20 hours.

This shows that Thy12 viability is severely impaired in the absence of thymidine.

**Figure 12:** Intestinal passage and viability: *L. lactis* MG1363 was transformed with the

5 plasmid pLET2N which carries a chloramphenicol (Cm) resistance marker. *L. lactis* Thy12 was transformed with the plasmid pT1NX which carries an erythromycin (Em) resistance marker. Of both strains 10<sup>9</sup> bacteria were resuspended in BM9 (6 g/l Na<sub>2</sub>HPO<sub>4</sub>, 3 g/l KH<sub>2</sub>PO<sub>4</sub>, 1 g/l NH<sub>4</sub>Cl, 0,5 g/l NaCl in 25 mM NaHCO<sub>3</sub> + 25 mM Na<sub>2</sub>CO<sub>3</sub>), mixed and inoculated in three mice at t=0h. Faeces were collected of the  
10 time intervals -1 to 0, 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, 5 to 6, 6 to 7, 7 to 8, 8 to 9, 9 to 10 and 10 to overnight. All samples were resuspended in isotonic buffer and appropriate dilutions were plated on GM17 (M17 medium, Difco, St.Louis supplemented with 0,5% glucose) plates containing either Cm, Em or Em+ 50µg/ml thymidine. Colony forming units for the different plates are represented in the graph.

15

### Description of the invention

It is the objective of the present invention to provide a suitable biological containment system for *Lactococcus*.

A first aspect of the invention is an isolated strain of *Lactococcus* sp. comprising a  
20 defective thymidylate synthase gene. Preferably, said defective thymidylate synthase gene is inactivated by gene disruption. Even more preferably, said *Lactococcus* sp. is *Lactococcus lactis*. A special embodiment is a *Lactococcus* sp. strain, preferably *Lactococcus lactis*, more preferably a *Lactococcus lactis* MG1363 derivative, whereby the thymidylate synthase gene has been disrupted and replaced by an interleukin-10  
25 expression unit. Said interleukin-10 expression unit is preferably, but not limited to, a human interleukin-10 expression unit or gene encoding for human interleukin-10.

Another aspect of the invention is the use of a strain according to the invention as host strain for transformation, whereby the transforming plasmid does not comprise an intact thymidylate synthase gene. Still another aspect of the invention is a transformed  
30 strain of *Lactococcus* sp. according to the invention, comprising a plasmid that does not comprise an intact thymidylate synthase gene. Another aspect of the invention relates to a transformed strain of *Lactococcus* sp. comprising a gene or expression unit encoding a prophylactic and/or therapeutic molecule such as interleukin-10. Consequently, the present invention also relates to the usage of a transformed strain

invention further demonstrates that the transformed strains surprisingly pass the gut at the same speed as the control strains and shows that their loss of viability is indeed not different from that of the control strains. However, once said strain is secreted in the environment, e.g. in the faeces, it is not able to survive any longer.

- 5 The transforming plasmid can be any plasmid, as long as it cannot complement the *thyA* mutation. It may be a selfreplicating plasmid that preferably carries one or more genes of interest and one or more resistance markers, or it may be an integrative plasmid. In the latter case, the integrative plasmid itself may be used to create the mutation, by causing integration at the *thyA* site, whereby the *thyA* gene is inactivated.
- 10 Preferably, the active *thyA* gene is replaced by double homologous recombination by a cassette comprising the gene or genes of interest, flanked by targeting sequences that target the insertion to the *thyA* target site. It is of extreme importance that these sequences are sufficiently long and sufficiently homologous to obtain to integrate the sequence into the target site. Preferably, said targeting sequences consist of at least
- 15 100 contiguous nucleotides of SEQ ID N°1 at one side of the gene of interest, and at least 100 contiguous nucleotides of SEQ ID N°2 at the other side; more preferably, said targeting sequences consists of at least 500 contiguous nucleotides of SEQ ID N°1 at one side of the gene of interest, and at least 500 contiguous nucleotides of the SEQ ID N° 2 at the other side; most preferably, said targeting sequences consists of
- 20 SEQ ID N°1 at one side of the gene of interest and SEQ ID N°2 at the other side, or said targeting sequences consist of at least 100 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 100 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of interest, preferably
- 25 said targeting sequences consist of at least 500 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 500 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of interest, most preferably said targeting sequences consist of at least 1000 nucleotides that are at
- 30 least 80% identical, preferably 90% identical to a region of SEQ ID N° 1 at one side of the gene of interest, and of at least 1000 nucleotides that are at least 80% identical, preferably 90% identical to a region of SEQ ID N° 2 at the other side of the gene of interest. The percentage identity is measured with BLAST, according to Altschul *et al.* (1997). A preferred example of a s quence, homologous to SEQ ID N°1 is given in

locus as determined in the present invention are given by SEQ ID N° 19, 20, 21, 22 respectively.

The *thyA* replacement is performed by making suitable replacements in a plasmid borne version of the *thyA* target, as described below. The carrier plasmid is a derivative of pORI19 (Law *et al.*, 1995) a replication defective plasmid, which only transfers the erythromycin resistance to a given strain when a first homologous recombination, at either the 5' 1000bp or at the 3'1000bp of the *thyA* target. A second homologous recombination at the 3' 1000bp or at the 5' 1000bp of the *thyA* target yields the desired strain.

The *thyA* gene is replaced by a synthetic gene encoding a protein which has the *L. lactis* Usp45 secretion leader (van Asseldonk *et al.*, 1990) fused to a protein of identical amino acid sequence than: (a) the mature part of human-interleukin 10 (hIL-10) or (b) the mature part of hIL-10 in which proline at position 2 had been replaced with alanine or (c) the mature part of hIL-10 in which the first two amino acids had been deleted; (a), (b) and (c) are called hIL-10 analogs, the fusion products are called Usp45-hIL-10.

The *thyA* gene is replaced by an expression unit comprising the lactococcal P1 promotor (Waterfield *et al.*, 1995), the *E. coli* bacteriophageT7 expression signals: putative RNA stabilising sequence and modified gene10 ribosomal binding site (Wells and Schofield, 1996).

At the 5' end the insertion is performed in such way that the ATG of *thyA* is fused to the P1-T7Usp45-hIL-10 expression unit.

5' agataggaaaattttc atg acttacgcagatcaagttttt...*thyA* wild type

gattaagtcacatcttacctctt...P1-T7-usp45-hIL10

5' agataggaaaattttc atg gattaagtcacatcttacctctt...*thyA*<sup>-</sup>, P1-T7-usp45-hIL10

Alternatively, at the 5' end the insertion is performed in such way that the *thyA* ATG is not included:

5' agataggaaaattttc acttacgcagatcaagttttt...*thyA* wild type

gattaagtcacatcttacctctt...P1-T7-usp45-hIL10

5' agataggaaaattttc gattaagtcacatcttacctctt...*thyA*<sup>-</sup>, P1-T7-usp45-hIL10

integrative plasmid pT1HIL10apxa. Figure 8 (panel A and B) further demonstrates that all mutants produce a significant amount of h-IL 10.

Figure 9 shows the production of hIL-10 by the *L. lactis* strains LL108 carrying either pOThy11, pOThy12, or pOThy16. Quantification (by ELISA) of hIL-10 present in the culture supernatant of the indicated strains. The N-terminal protein sequence of the recombinant hIL-10 was determined by Edman degradation and was shown identical to the structure as predicted for the mature, recombinant hIL-10. The protein showed full biological activity. LL108 is a *L. lactis* strain carrying a genomic integration of the repA gene, required for replication of pORI19 derived plasmids such as pOThy11, pOThy12, pOThy15 or pOThy16. This strain was kindly donated by dr. Jan Kok, University of Groningen. The plasmids pOThy11, pOThy12, pOThy15 and pOThy16 carry the synthetic human IL-10 gene in different promotor configurations (see Fig. 2), flanked by approximately 1kB of genomic DNA derived from the thyA locus, upstream and downstream from thyA. These plasmids were used for the construction of the genomic integration as described.

The effect of the thymidilate synthase deletion on the growth in thymidine less and thymidine supplemented media was tested; the results are summarized in figures 10 and 11. Absence of thymidine in the medium strongly limits the growth of the mutant, and even results in a decrease of colony forming units after four hours of cultivation. Addition of thymidine to the medium results in an identical growth curve and amount of colony forming units, compared to the wild type strain, indicating that the mutant doesn't affect the growth or viability in thymidine supplemented medium. Fig. 11 clearly demonstrates that Thy12 viability is severely impaired in the absence of thymidine.

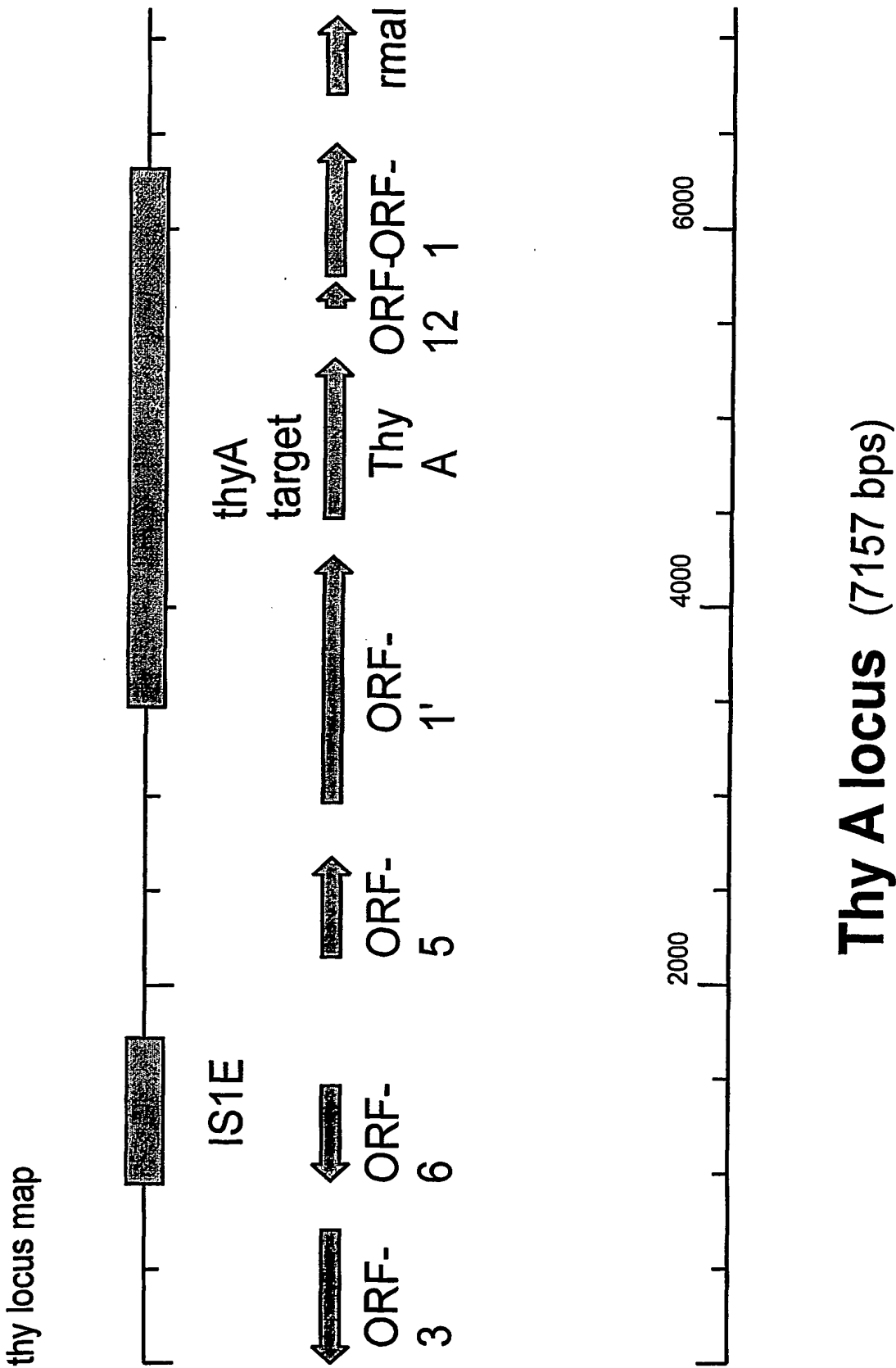
Fig. 12 finally shows that *L. lactis* Thy12 passes the intestine of the mice at the same speed as MG1363. Loss of viability does not appear different between Thy12 and MG1363. Thy12 appear fully dependent on thymidine for growth, indicating that no Thy12 bacteria had taken up a foreign thyA gene.

**Claims**

1. An isolated strain of *Lactococcus* sp. comprising a defective thymidylate synthase gene.
2. An isolated strain of *Lactococcus* sp. according to claim 1, whereby said gene is inactivated by gene disruption.
3. An isolated strain of *Lactococcus* sp. according to claim 1 or 2, whereby said *Lactococcus* sp. is *Lactococcus lactis*.
4. The use of a strain of *Lactococcus* sp. according to any of the claims 1-3 as host strain for transformation, whereby the transforming plasmid does not comprise an intact thymidylate synthase gene.
5. A transformed strain of *Lactococcus* sp. according to any of the claims 1-3, comprising a transforming plasmid that does not comprise an intact thymidylate synthase gene.
6. A transformed strain of *Lactococcus* sp. according to any of the claims 1-4 comprising a gene encoding a prophylactic and/or therapeutic molecule.
7. A transformed strain of *Lactococcus* sp. according to claim 6 wherein said prophylactic and/or therapeutic molecule is interleukin-10.
8. The use of a transformed strain of *Lactococcus* sp. according to any of the claims 5-7 for the delivery of prophylactic and/or therapeutic molecules.
9. A pharmaceutical composition comprising a transformed strain of *Lactococcus* sp. according to any of the claims 5-7.
10. The use of a transformed strain of *Lactococcus* sp. according to any of the claims 6-7 for the preparation of a medicament to treat inflammatory bowel diseases.



Figure 1:



2/9

Figure 2:

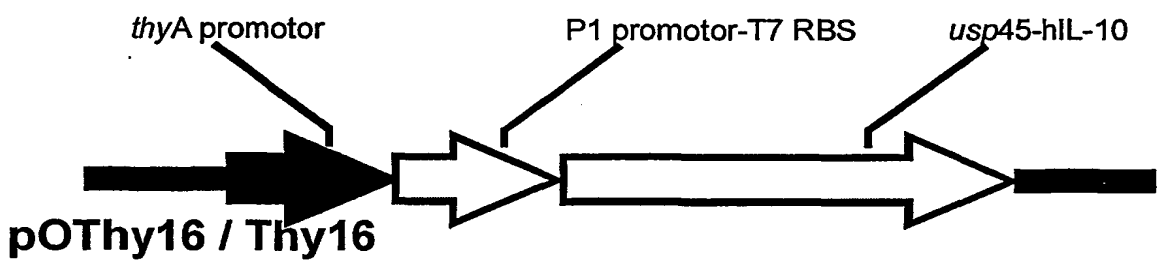
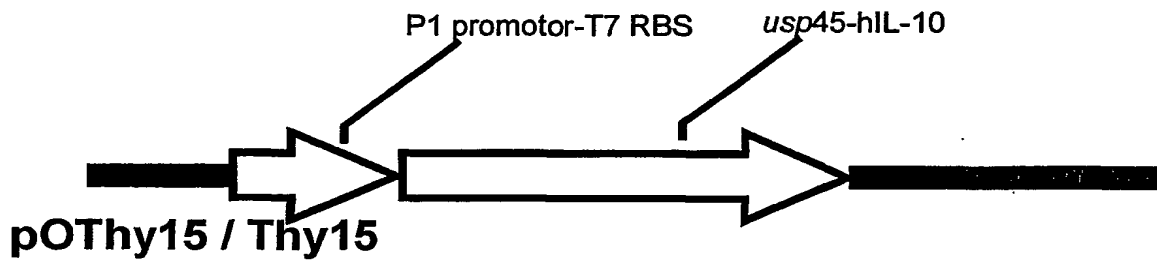
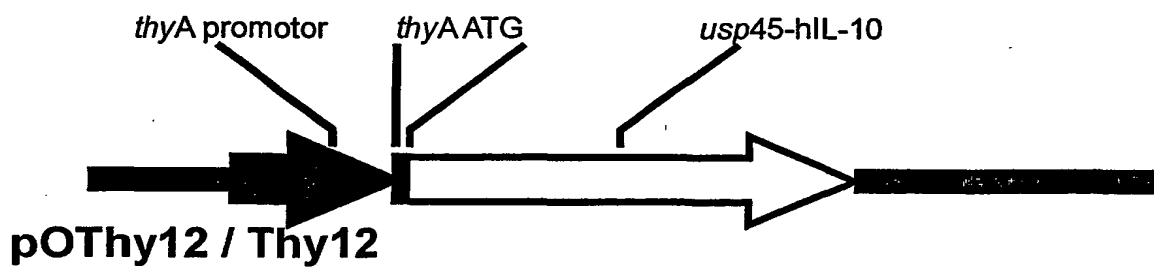
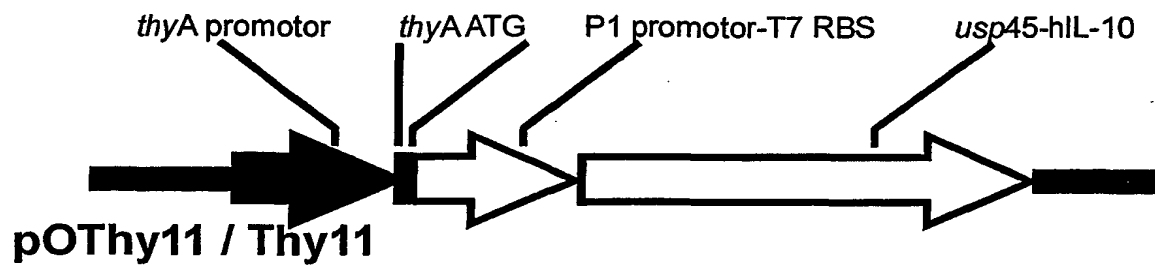
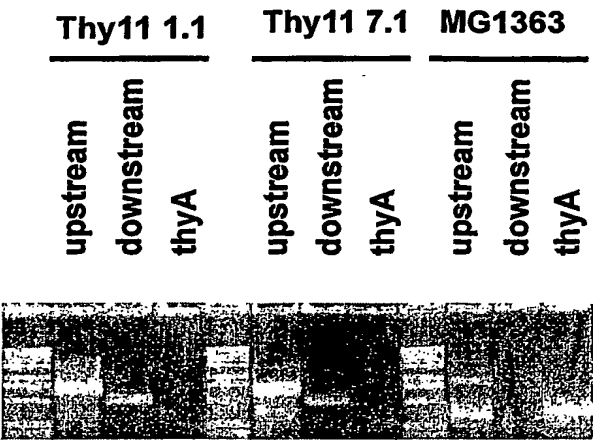
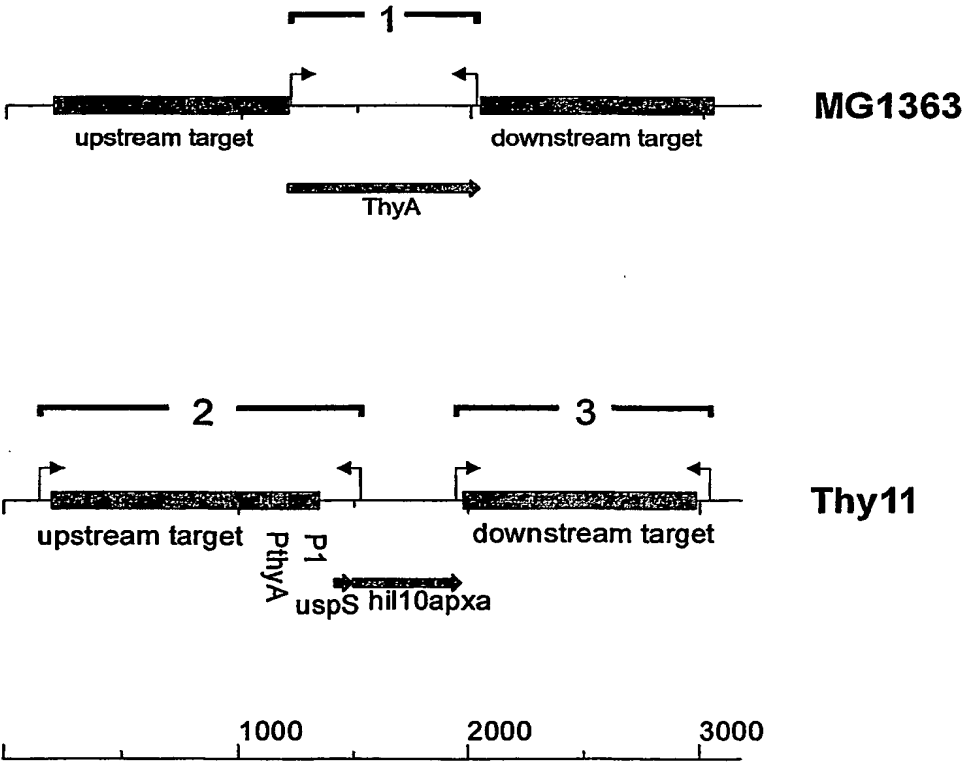


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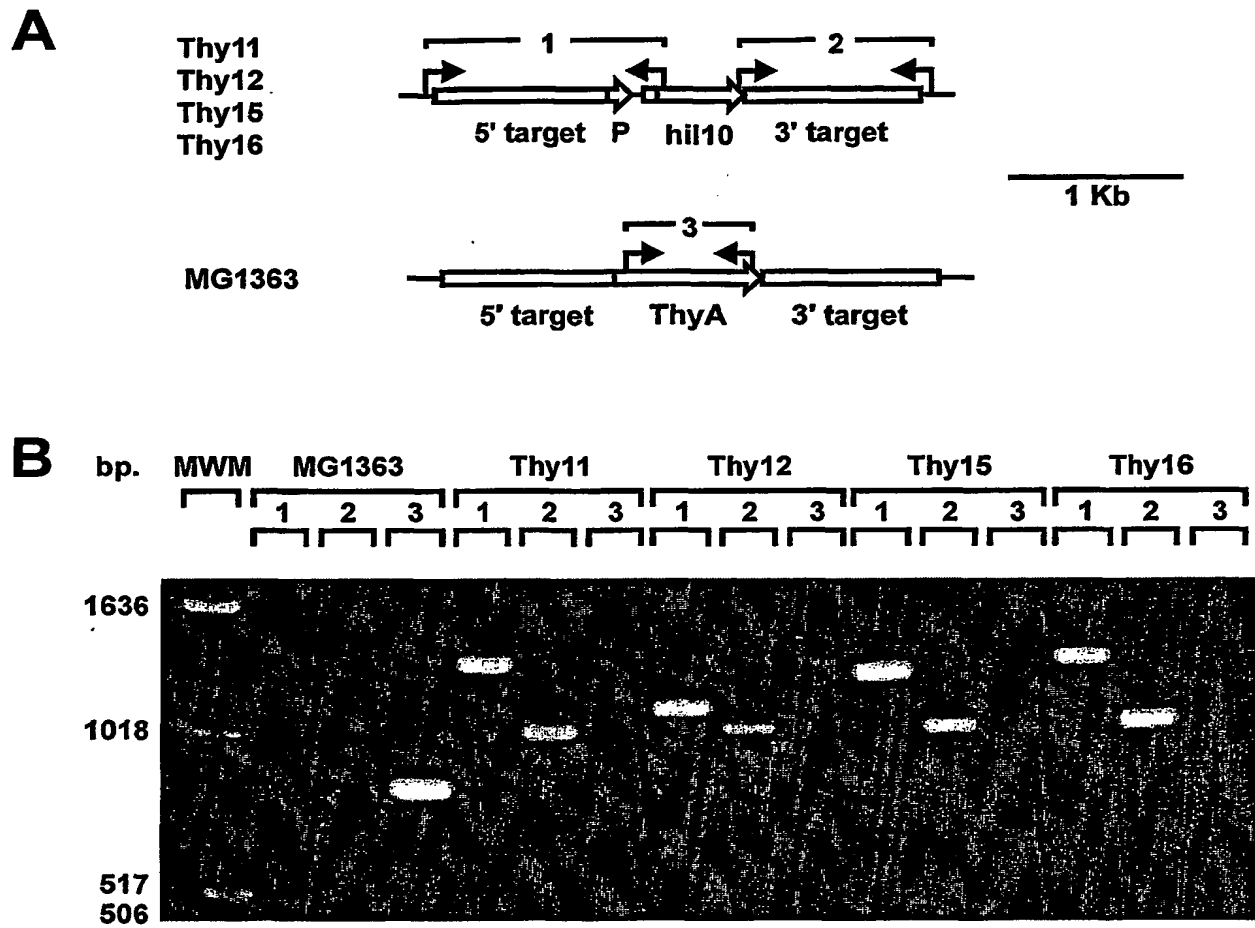


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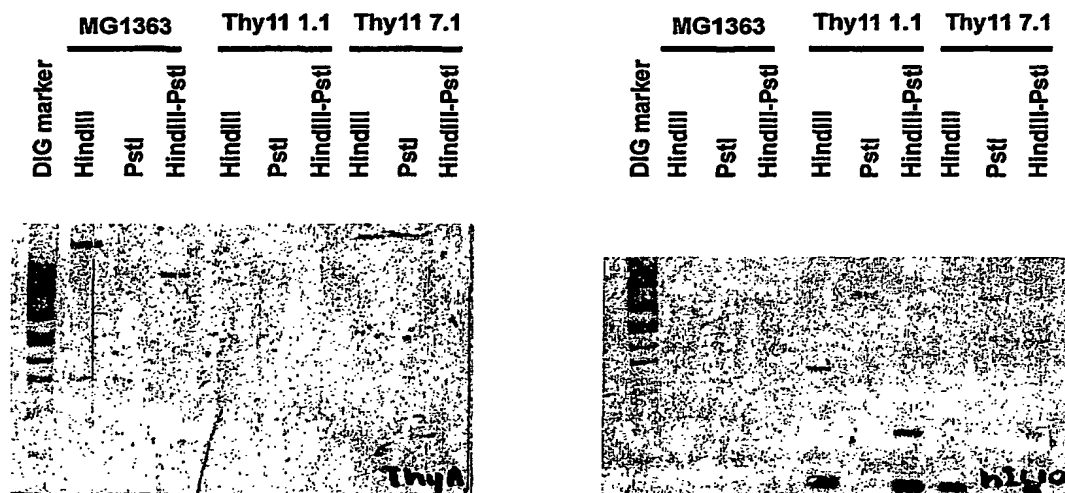
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5/9

Figure 5:

A



6/9

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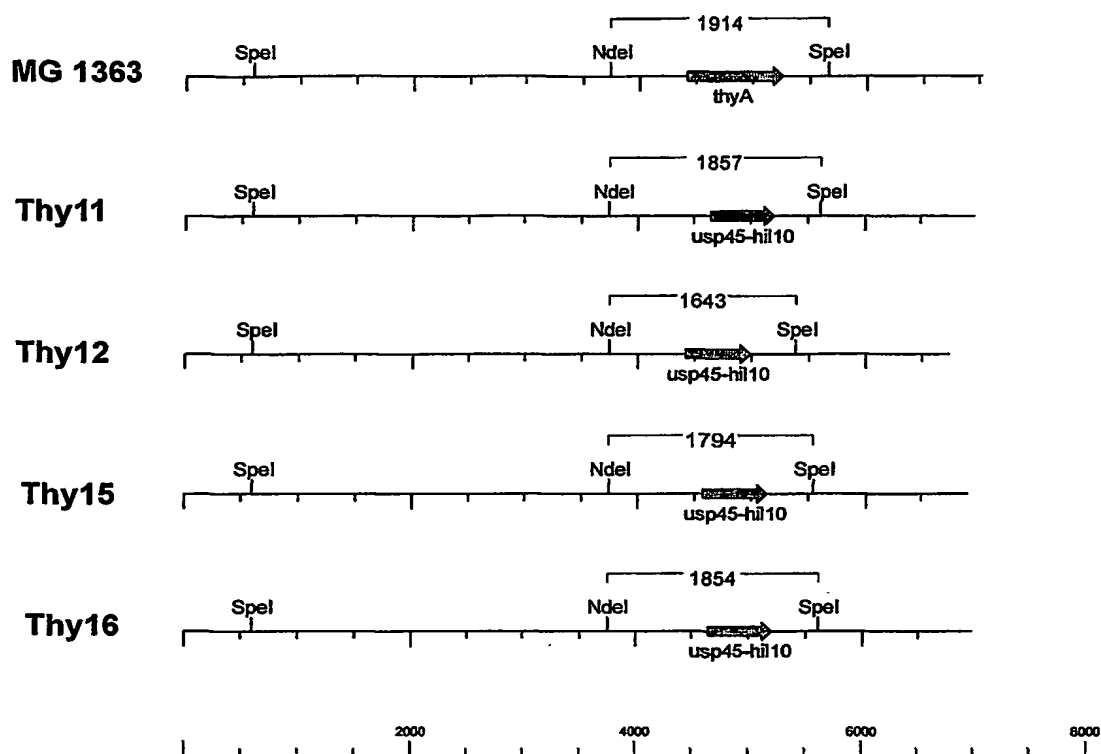
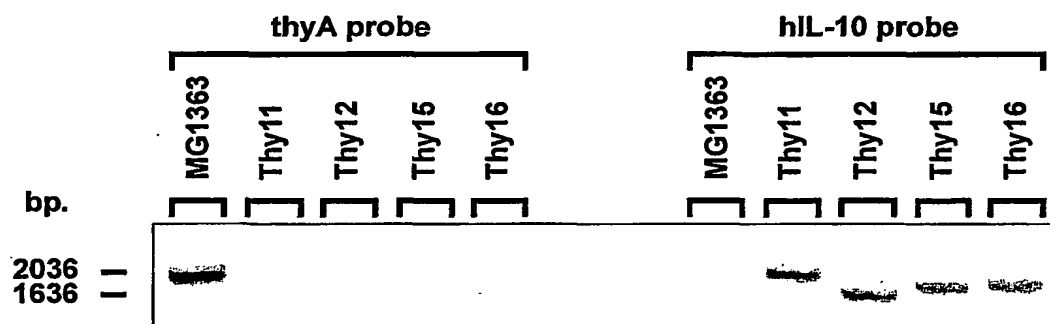
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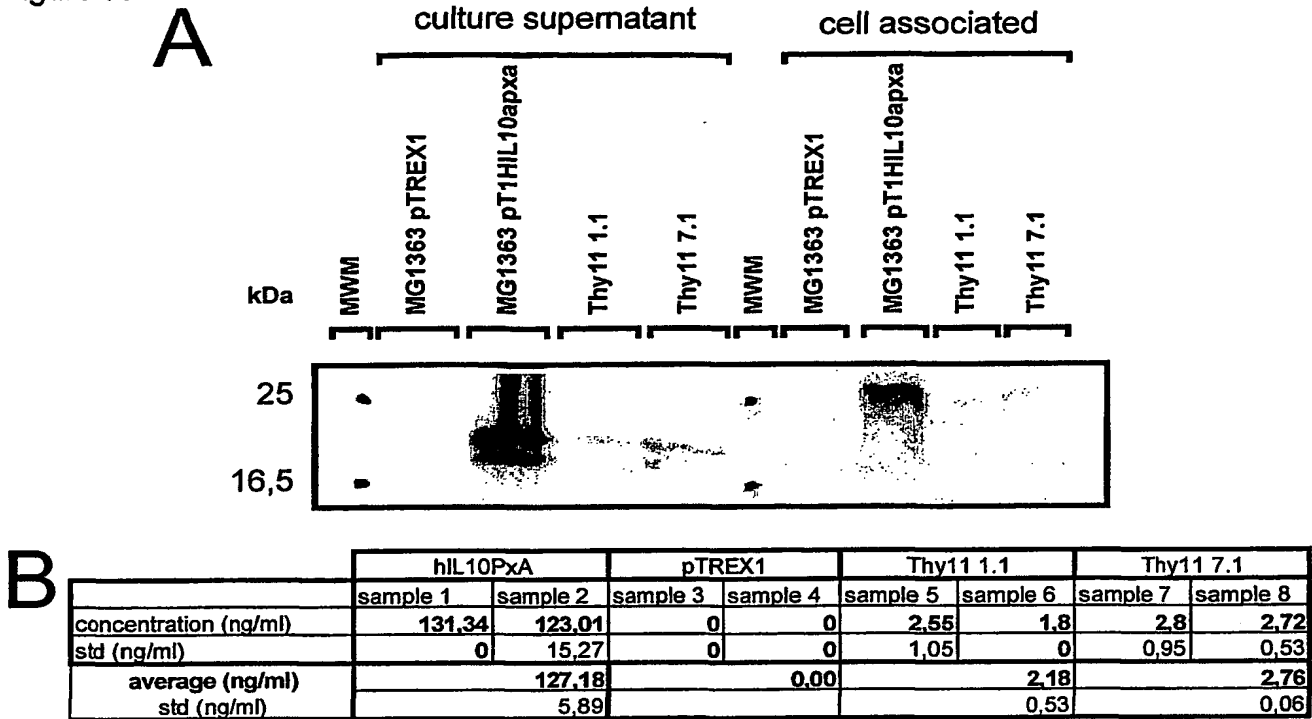
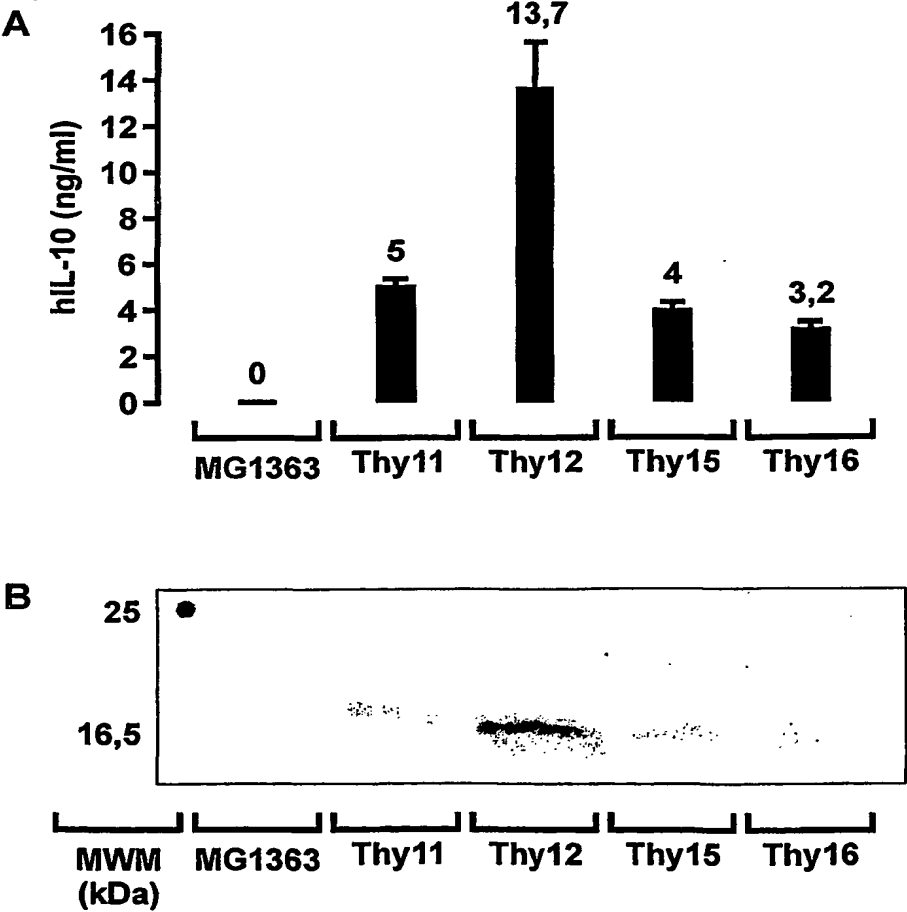


Figure 8:



8/9

Figure 9:

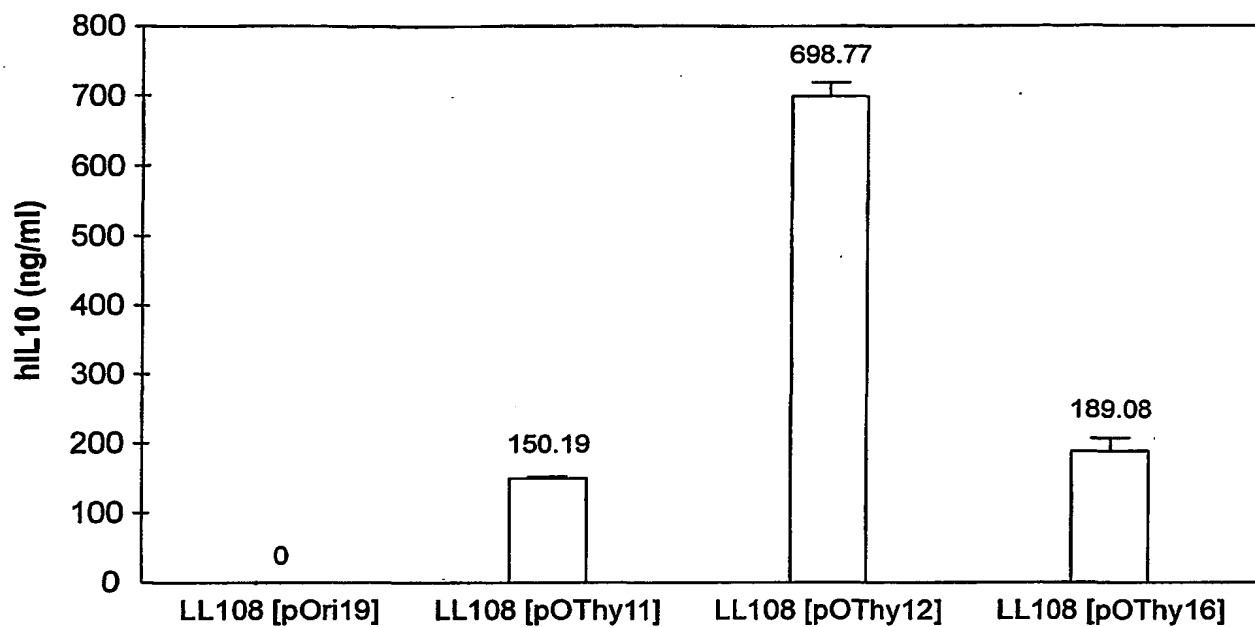
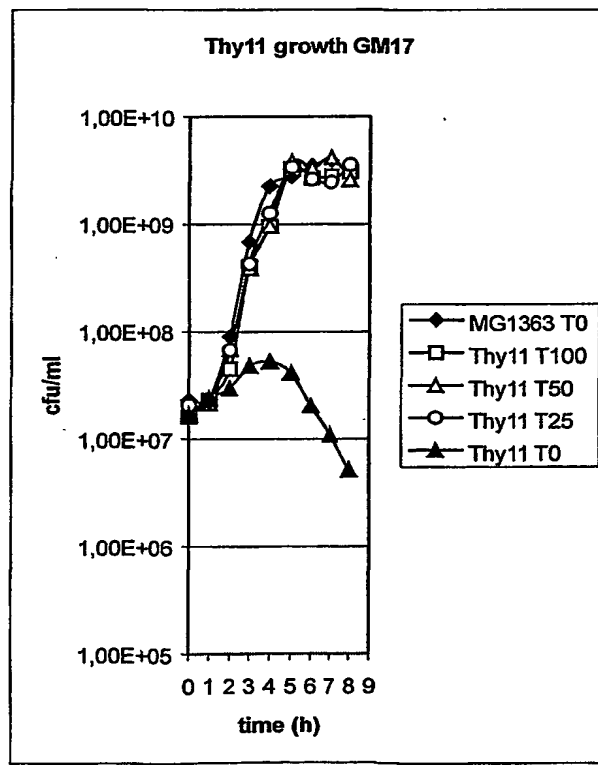
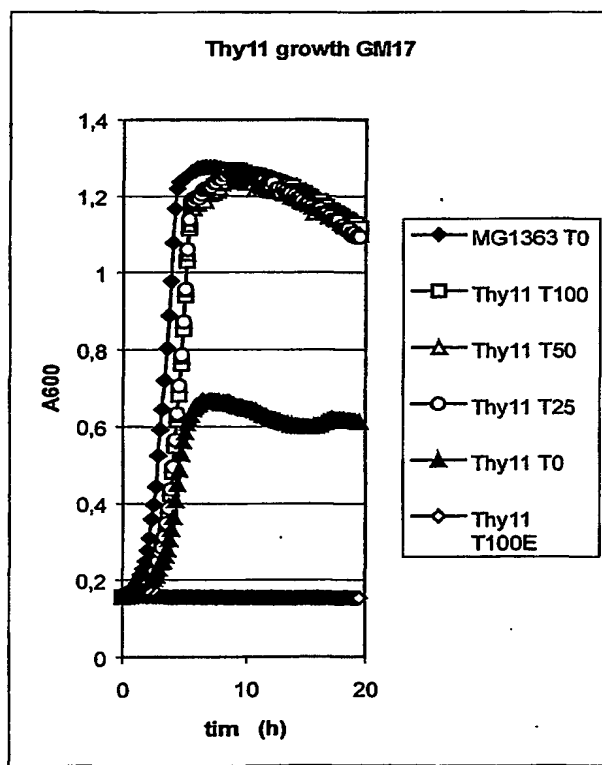


Figure 10:





9/9

Figure 11:

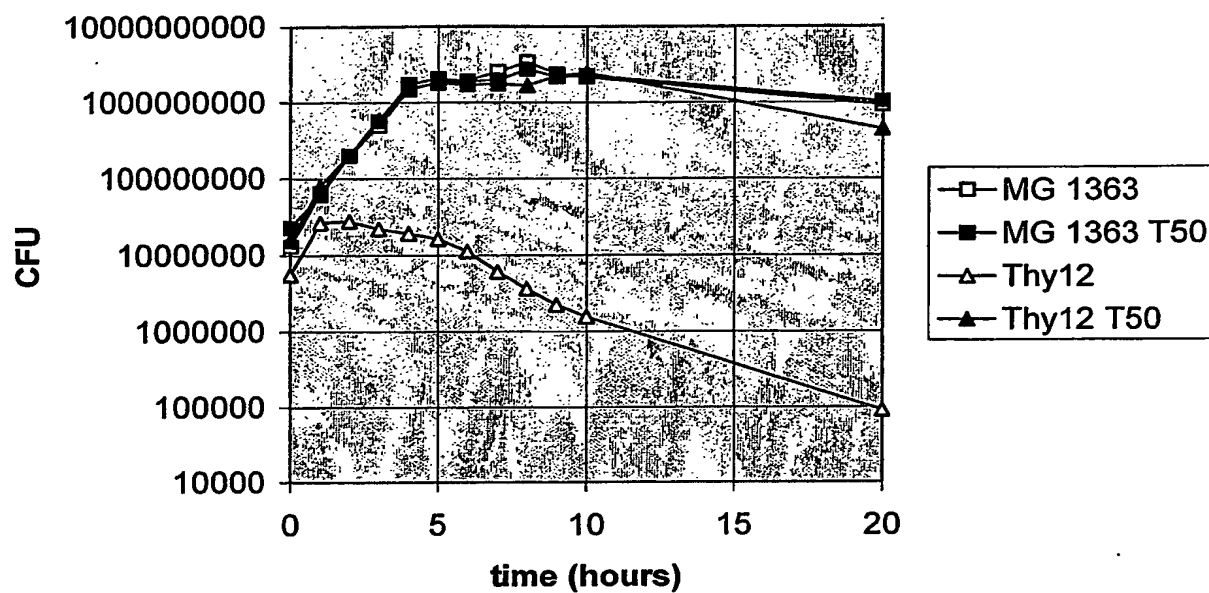
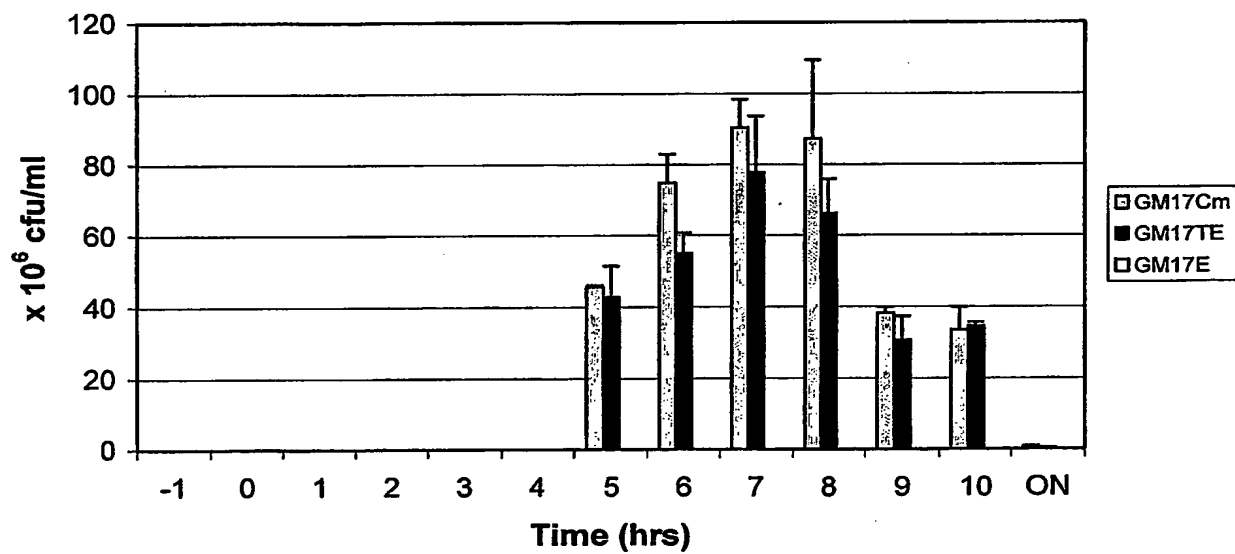


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 ccgattttga gaacgacagc gacttccgtc ccagccgtgc caggtgctgc ctcagattca 1080  
 ggttatgccg ctcaattcgc tgcgtatatc gcttgctgat tacgtgcagc tttcccttca 1140  
 ggcgggattc atacagcggc cagccatccg tcatccatat caccacgtca aagggtgaca 1200  
 gcaggctcat aagacgccc agcgtcgcca tagtgcggtc accgaatacg tgcgcaacaa 1260  
 ccgtcttccg gagactgtca tacgcgtaaa acagccagcg ctggcgcgat ttagccccga 1320

## V085.ST25.txt

```

gaaatccgcg gtagttgaca gtgtgtcaaa tggtgaagca tttcaaacgg tatacacggg 3420
tagcacagga ttaattgtag caatcataat tggttttatt gtttcattag tctatataca 3480
attgagcaaa agaaatttag ttattaaatt accagctgga gttcctccaa tggttgtaga 3540
ttcactaagt ccagcaatta tttcaatggg gattttctgt ttgatgttcg ggattcgtgt 3600
gggattctct tatacgccat tccatgatat tttcaatttc tcaacacaac taattcaagc 3660
accgttgact ggtgctgtgg caaatccatg ggttcttatg ggcatcttta cctttggtaa 3720
tttcttatgg ttctttggta tccaccctaa tttaattggg ggaattttta atccattggt 3780
attaacaatg tcatatgcta atattgatgc ctatgctgcc ggaaaacctg taccatactt 3840
acaaatgatg attgtgtttg ctgtgggtgc gaacgcattg ggcggaagtg gaaatactta 3900
tgggttagtt atttcaatgt ttacggcaaa atctgaacgc tataaacaat tattaaaatt 3960
aggtgcaatt cctagtatct tcaatatcag tgaaccatta ctttttggtc ttccaatgat 4020
gttaaatcct cttttcttta ttcttttggg tttccaacca gcaatttttag gaactgtagc 4080
attgggcttg gcaaagatat tatataattac aaatctgaat ccaatgacgg cacttcttcc 4140
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taataaagct ttagaagaag aaaaagcagc tggtgaatta gaggggttcag aaactgcctg 4320
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gataattctg agaggttatt ttgggaaata ctattgaacc atatcgaggt gtgtggtata 4440
atgaagggaa ttaaaaaaga taggaaaatt tc atg act tac gca gat caa gtt 4493
                Met Thr Tyr Ala Asp Gln Val
                1                5

ttt aaa caa aat atc caa aat atc cta gat aat ggt gtt ttt tca gaa 4541
Phe Lys Gln Asn Ile Gln Asn Ile Leu Asp Asn Gly Val Phe Ser Glu
                10                15                20

aat gca aga cca aag tat aag gat ggt caa atg gcg aat agc aaa tat 4589
Asn Ala Arg Pro Lys Tyr Lys Asp Gly Gln Met Ala Asn Ser Lys Tyr
                25                30                35

gtc act ggt tca ttc gtt act tat gat ttg caa aag ggg gag ttt cca 4637
Val Thr Gly Ser Phe Val Thr Tyr Asp Leu Gln Lys Gly Glu Phe Pro
40                45                50                55

att acc act ttg cgt cca att cca atc aaa tct gct att aaa gaa ttg 4685
Ile Thr Thr Leu Arg Pro Ile Pro Ile Lys Ser Ala Ile Lys Glu Leu
                60                65                70

atg tgg ata tac caa gac caa aca agt gaa ctt tct gtt ctc gaa gag 4733
Met Trp Ile Tyr Gln Asp Gln Thr Ser Glu Leu Ser Val Leu Glu Glu
                75                80                85

aag tat gga gtc aaa tac tgg gga gaa tgg gga att ggt gat ggt acg 4781
Lys Tyr Gly Val Lys Tyr Trp Gly Glu Trp Gly Ile Gly Asp Gly Thr
90                95                100

att ggg caa cgt tat ggt gca aca gtc aaa aaa tat aat atc att ggt 4829
Ile Gly Gln Arg Tyr Gly Ala Thr Val Lys Lys Tyr Asn Ile Ile Gly
105                110                115

```

## V085.ST25.txt

```

cttgaaatta atgcaatggg aaatcttact ttaatatgga aaggggcaaa gaatcaaacc 6202
tttgaacttg ggcgagggtca acaatttaac ggaactgcag atattgcctt aaaaaatgga 6262
gagattttccc ctggtagtcc acttaacatt tttgttgtac caacagaagt tgctttccct 6322
aataataaaa aagtagacga ttcaactggg caacaacgaa tttttgtgaa ttattctggt 6382
acaagccctc aaatggcgaa tagtatggca gcggtggctt tttttagagt tattccatga 6442
ttatattaaa gttagaattg aataaaatgt attattaaaa agataatatt atatcacgac 6502
aaggcgacat ctatcaactt taccactggt atggaagtga ccattattac atcaggaaac 6562
gctaaaacgg ttgtttttac acccgtaaaa taaataataa aataatgtgn aattactgac 6622
agcattttgt cagtaatttt ttttatcaaa atcacacaaa aatgttcgtt gacgaacaaa 6682
aaaaactatg ttataataat tcgtatgcga actaaaaaag aagcgattgg ccgactttta 6742
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acaggtcagc aaatgtcaat ttttagatttt cttggaaatc aaagcgaaga aggttcagga 6862
aaagaaatta gtcagacgat gattgaatta gaatttaata tccgacgttc aacaacgacg 6922
gaaattttac agcgcattga aaagcggtt ttaattaatc gaagaacaag cctgaccgat 6982
gcccgccaaa aatcagttga attaactgaa gaagggaaaa gatatttacc tgaaatcagg 7042
gcttatatcc aagcacataa taaaaaagct tggcgtaatc atgggtcatag ctgtttncct 7102
gggttagngg gccannnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn cnnnc 7157

```

```

<210> 4
<211> 279
<212> PRT
<213> Lactococcus lactis

```

```

<220>
<221> misc_feature
<222> (2)..(2)
<223> 'n' may be any base

```

```

<220>
<221> misc_feature
<222> (5)..(5)
<223> 'n' may be any base

```

```

<220>
<221> misc_feature
<222> (6612)..(6612)
<223> 'n' may be any base

```

```

<220>
<221> misc_feature
<222> (7099)..(7099)
<223> 'n' may be any base

```

```

<220>
<221> misc_feature
<222> (7110)..(7110)
<223> 'n' may be any base

```

```

<220>
<221> misc_feature
<222> (7117)..(7141)

```

## V085.ST25.txt

Phe Glu Gln Ala Asn Glu Leu Met Lys Arg Thr Ala Ser Glu Lys Glu  
225 230 235 240

Pro Arg Leu Val Leu Asn Val Pro Asp Gly Thr Asn Phe Phe Asp Ile  
245 250 255

Lys Pro Glu Asp Phe Glu Leu Val Asp Tyr Glu Pro Val Lys Pro Gln  
260 265 270

Leu Lys Phe Asp Leu Ala Ile  
275

<210> 5  
<211> 7094  
<212> DNA  
<213> Lactococcus lactis

<220>  
<221> CDS  
<222> (4469) .. (5305)  
<223>

<400> 5  
ggttttccca gtccgacggt gtaaaacgac ggccagtga ttcattaaca gccttttgag 60  
cagctagctc attattttga aataaatcat aaatttcttt cccactatct gatttatgat 120  
tgctagcata tttgttgtat aatcgaacga gtccattttg aacagatcca tatagattga 180  
gtgaactata aaatacatct atatcatagt tgagtttggt cacaatcatg agaccaaatt 240  
ctccagcatt tcgtgtagaa ccacgataaa gctgtttatt tagcaaaatg gcacctccga 300  
cacctgtacc taaagtcatg caaataaaat tttggctttc ttgtccattc cctagccaaa 360  
gttcagctag acctgcacaa ttggcatcat tttcaacata aaccggaaga tttaaatggt 420  
tttgtagttc tgtccccaat ggatagccat aaagatcagt tagagctcct gccagtaata 480  
atgttccctt tttgtcagaa gttccgggaa cacttacacc aattgcagat actgaatgat 540  
gagcttttaa ctgatgaata tttgtgagca agctatccat aattttttct ttttttaatg 600  
gggttggaac ttgtaaatgt tgtatgatcg ttccatcact agttacaaga ccaaatttta 660  
taaattgtacc accgatatca attcctattg aataatgcat cttttattac ctctttctct 720  
aatttgtttt agtatagcaa aatcaaaaaa ttaattatgg tatgcattat agatatgttg 780  
tataattttc acaaaaacgg agaaaactat gaaaacaata gaacagctca tgatagattc 840  
agcagattta atgtcagatt ttattcaatt gacaattttt atattccgca aggaggattt 900  
tcaacttttt tataggagtg atgaagaaga gcaagctttt tcaaggtaat gactccaact 960  
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tttgagaacg acagcgactt ccgtcccagc cgtgccaggt gctgcctcag attcagggtta 1080  
tgccgctcaa ttcgctgcgt atatcgcttg ctgattacgt gcagctttcc cttcaggcgg 1140

V085.ST25.txt

gtgcagttac cagtgcgcta gcaatttatg taacttataa ttttgcttat tcttatgtaa	3240
atcgatcatga atataatggc catacggccg gtttattatc aatcgcaagt ttgttaatgc	3300
taatgccaca aattattact gtccctgtag taaaaaacat tccaaccgaa tttccgaaat	3360
ccgcggtagt tgacagtgtg tcaaagtgtg aagcatttca aacgggtatac acgggtagca	3420
caggattaat tgtagcaatc ataattgggt ttattgtttc attagtctat atacaattga	3480
gcaaaagaaa tttagttatt aaattaccag ctggagttcc tccaatgggt gtagattcac	3540
taagtccagc aattatttca atgggtgatt tctgtttgat gtccgggatt cgtgtgggat	3600
tctcttatac gccattccat gatattttca atttctcaac acaactaatt caagcaccgt	3660
tgactgggtgc tgtggcaaat ccatgggttc ttatgggcat ctttaccttt ggtaatttct	3720
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caatgtcata tgctaataat gatgcctatg ctgccggaaa acctgtacca tacttacaaa	3840
tgatgattgt gtttgctgtg ggtgcgaacg catggggcgg aagtggaaat acttatgggt	3900
tagttatttc aatgtttacg gcaaaatctg aacgctataa acaattatta aaattaggtg	3960
caattcctag tatttttcaat atcagtgaac cattactttt tggctctcca atgatgttaa	4020
atcctctttt ctttattcct ttggttttcc aaccagcaat tttaggaact gtagcattgg	4080
gcttggcaaa gatattatat attacaaatc tgaatccaat gacggcactt cttccttgga	4140
cgacaccagc acctgtgaga atggccattt cagggtggact tccatttttg attatttttg	4200
caatctgttt agtcttgaat gttcttattt actaccattt ctttaagggtg gcgtataata	4260
aagctttaga agaagaaaaa gcagctgttg aattagaggg ttcagaaact gcctgatgga	4320
tattttttat aaatctgggt tgaacaaatt atattgacat ctctttttct atcctgataa	4380
ttctgagagg ttattttggg aaatactatt gaaccatatac gaggtgggtg ggtataatga	4440
aggggaattaa aaaagatagg aaaatttc atg act tac gca gat caa gtt ttt	4492
Met Thr Tyr Ala Asp Gln Val Phe	
1 5	
aaa caa aat atc caa aat atc cta gat aat ggt gtt ttt tca gaa aat	4540
Lys Gln Asn Ile Gln Asn Ile Leu Asp Asn Gly Val Phe Ser Glu Asn	
10 15 20	
gca aga cca aag tat aag gat ggt caa atg gcg aat agc aaa tat gtc	4588
Ala Arg Pro Lys Tyr Lys Asp Gly Gln Met Ala Asn Ser Lys Tyr Val	
25 30 35 40	
act ggt tca ttc gtt act tat gat ttg caa aag ggg gag ttt cca att	4636
Thr Gly Ser Phe Val Thr Tyr Asp Leu Gln Lys Gly Glu Phe Pro Ile	
45 50 55	
acc act ttg cgt cca att cca atc aaa tct gct att aaa gaa ttg atg	4684
Thr Thr Leu Arg Pro Ile Pro Ile Lys Ser Ala Ile Lys Glu Leu Met	
60 65 70	
tgg ata tac caa gac caa aca agt gaa ctt tct gtt ctc gaa gag aag	4732
Trp Ile Tyr Gln Asp Gln Thr Ser Glu Leu Ser Val Leu Glu Glu Lys	
75 80 85	
tat gga gtc aaa tac tgg gga gaa tgg gga att ggt gat ggt acg att	4780
Tyr Gly Val Lys Tyr Trp Gly Glu Trp Gly Ile Gly Asp Gly Thr Ile	

V085.ST25.txt

```

attacacgga aagtagcttt gagcaaaata gcttattgaa tactgggttg aaatatgggg 6025
cagtagcttg gtacgggatt ggagtaaaaa acgaaatgtt aaacattgct caaattgtta 6085
gtggtaattt ttctagtatt gttggaactt ggaaagatac ttctggaaat atgcttgaaa 6145
ttaatgcaat gggaaatctt actttaatat ggaaaggggc aaagaatcaa acctttgaac 6205
ttggcgcagg tcaacaattt aatggaactg cagatattgc cttaaaaaat ggagagattt 6265
cccctggtag tccacttaac atttttgttg taccaacaga agttgctttc cctaataata 6325
aaaaagtaga cgattcaact gggcaacaac gaatttttgt gaattattct ggtacaagcc 6385
ctcaaattggc gaatagtatg gcagcggtag ctttttttag agttattcca tgattatatt 6445
aaagttagaa ttgaataaaa tgtattatta aaaagataat attatatcac gacaaggcga 6505
catctatcaa ctttaccact ggtatggaag tgaccattat tacatcagga aacgctaaaa 6565
cggttgtttt tacacccgta aaataaataa taaaataatg tgaaattact gacagcattt 6625
tgtcagtaat tttttttatc aaatcacac aaaaatgttc gttgacgaac aaaaaaact 6685
atgttataat aattcgtatg cgaactaaaa agaagcgat tggccgactt taaaagtag 6745
ccagcaacca aatgtctcga gaatttgata attttgcagc tcaacttgat ttgacaggtc 6805
agcaaattgtc aatttttagat tttcttgga atcaaagcga agaagggttca ggaaaagaaa 6865
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tacagcgcac ggaaaagcgg cttttaatta atcgaagaac aagcctgacc gatgcccgcc 6985
aaaaatcagt tgaattaact gaagaaggga aaagatattt acctgaaatc agggcttata 7045
tccaagcaca taataaaaaa gcttggcgta atcatggtca tagctgttt 7094

```

<210> 6  
 <211> 279  
 <212> PRT  
 <213> Lactococcus lactis

<400> 6

Met Thr Tyr Ala Asp Gln Val Phe Lys Gln Asn Ile Gln Asn Ile Leu  
 1 5 10 15

Asp Asn Gly Val Phe Ser Glu Asn Ala Arg Pro Lys Tyr Lys Asp Gly  
 20 25 30

Gln Met Ala Asn Ser Lys Tyr Val Thr Gly Ser Phe Val Thr Tyr Asp  
 35 40 45

Leu Gln Lys Gly Glu Phe Pro Ile Thr Thr Leu Arg Pro Ile Pro Ile  
 50 55 60

Lys Ser Ala Ile Lys Glu Leu Met Trp Ile Tyr Gln Asp Gln Thr Ser  
 65 70 75 80

Glu Leu Ser Val Leu Glu Glu Lys Tyr Gly Val Lys Tyr Trp Gly Glu  
 85 90 95



## V085.ST25.txt

```

catacttaca aatgatgatt gtgtttgctg tgggtgcgaa cgcattggggc ggaagtggaa      420
atacttatgg gttagttatt tcaatgttta cggcaaaatc tgaacgctat aaacaattat      480
taaaattagg tgcaattcct agtatatttca atatcagtga accattactt tttgggtcttc      540
caatgatgtt aaatcctctt ttctttattc ctttgggtttt ccaaccagca attttaggaa      600
ctgtagcatt gggcttgga aagatattat atattacaaa tctgaatcca atgacggcac      660
ttcttccttg gacgacacca gcacctgtga gaatggccat ttcaggtgga cttccatttt      720
tgattatttt tgcaatctgt ttagtcttga atgttcttat ttactacca ttctttaagg      780
tggcgtataa taaagcttta gaagaagaaa aagcagctgt tgaattagag gggttcagaaa      840
ctgcctgatg gatatttttt ataaatctgg tttgaacaaa ttatattgac atctcttttt      900
ctatcctgat aattctgaga gggtattttt ggaaatacta ttgaaccata tcgaggtggt      960
gtggtataat gaagggaatt aaaaaagata ggaaaatttc      1000

```

```

<210> 8
<211> 24
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> oligonucleotide primer

```

```

<400> 8
atgacttacg cagatcaagt tttt      24

```

```

<210> 9
<211> 27
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> oligonucleotide primer

```

```

<400> 9
ttaaattgct aaatcaaatt tcaattg      27

```

```

<210> 10
<211> 20
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> oligonucleotide primer

```

```

<400> 10
tctgattgag taccttgacc      20

```

```

<210> 11
<211> 22
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> oligonucleotide primer

```

```

<400> 11

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V085.ST25.txt

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; thyA promoter not included, theA-, P1-T7-usp45-hIL10

&lt;400&gt; 17

tctgagaggt tatttttgga aatactagat taagtcacat tacctctt

48

&lt;210&gt; 18

&lt;211&gt; 40

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; thyA-, usp45-hIL10

&lt;400&gt; 18

aaaatccgta actaactaga attaatctat aagttactga

40

&lt;210&gt; 19

&lt;211&gt; 6967

&lt;212&gt; DNA

&lt;213&gt; Lactococcus lactis

&lt;220&gt;

&lt;221&gt; misc feature

&lt;223&gt; Thy11

&lt;400&gt; 19

attaacagcc ttttgagcag ctagctcatt attttgaaat aaatcataaa tttctttccc	60
actatctgat ttatgattgc tagcatatth gttgtataat cgaacgagtc cattttgaac	120
agatccatat agattgagtg aactataaaa tacatctata tcatagttga gtttgttcac	180
aatcatgaga ccaaattctc cagcatttcg tgtagaacca cgataaagct gtttatttag	240
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tgcagatact gaatgatgag cttttaactg atgaatattt gtgagcaagc tatccataat	540
tttttctttt tttaatgggg ttggaacttg taaatgttgt atgatcgttc catcactagt	600
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catgcagctc caccgatttt gagaacgaca gcgacttccg tcccagccgt gccaggtgct	1020
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## V085.ST25.txt

tttttcagca gttattggtg cagttaccag tgcgctagca atttatgtaa cttataat	3180
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cgcaagtttg ttaatgctaa tgccacaaat tattactgtc cctgtagtaa aaaacattcc	3300
aaccgaat	3360
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agtctatata caattgagca aaagaaat	3480
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at	4200
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caaagctcac gttaactcat tgggtgaaaa ccttaaaact ttgcgtcttc gtttgcgctg	5040
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ttttaacaaa ttgcaagaaa aaggtatcta caaagctatg tcagaatttg atatctttat	5160

## V085.ST25.txt

&lt;220&gt;

&lt;221&gt; misc feature

&lt;223&gt; Thy12

&lt;400&gt; 20

```

attaacagcc ttttgagcag ctagctcatt attttgaaat aaatcataaa tttctttccc      60
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## V085.ST25.txt

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V085.ST25.txt

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V085.ST25.txt

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## V085.ST25.txt

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## V085.ST25.txt

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## V085.ST25.txt

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